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Molecular Docking Analysis of Antifreeze Proteins with Lysophosphatidylcholine from Buffalo Sperm: Structural Insights for Cryopreservation

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ABSTRACT

Antifreeze proteins (AFPs) are specialized biomolecules that enable survival under subzero conditions by inhibiting ice crystal formation and minimizing cryoinjury. In the context of reproductive cryobiology, AFPs have garnered interest for their potential to protect cellular membranes during freezing, particularly in spermatozoa. This study explores the molecular interaction between AFPs and lysophosphatidylcholine (LPC), a major phospholipid constituent of buffalo (Bubalus bubalis) sperm membranes. Using molecular docking simulations with AutoDock 4.2, followed by structural visualization in PyMOL and energy optimization via Gaussian 09 (PM6 method), ten AFP isoforms were computationally docked with LPC. Results revealed favorable binding energies in several AFP–LPC complexes, especially in type II AFPs (e.g., 2AFP: -86.53 kcal/mol) and winter flounder AFP (1WFA: -47.71 kcal/mol). Ligand binding predominantly occurred within hydrophilic grooves and amphipathic clefts, mimicking membrane orientation and supporting structural compatibility. These findings suggest a dual mechanism of AFP action external ice inhibition and internal lipid stabilization, underscoring their utility as membrane-targeting cryoprotectants for improving post-thaw sperm viability in buffalo and other sensitive livestock species..

INTRODUCTION

Cryopreservation of mammalian spermatozoa plays a pivotal role in reproductive biotechnology, particularly in the conservation of valuable livestock genetics (Akhtar et al., 2022). However, sperm cells especially those from buffalo (*Bubalusbubalis*) are notoriously susceptible to cryodamage due to their unique membrane composition. The biophysical insult during freezing and thawing, such as osmotic shock, membrane destabilization, and oxidative stress, undermines sperm viability, motility, and ultimately fertility rates (Vale et al., 2022). These issues are largely attributed to the loss of membrane fluidity and the peroxidation of polyunsaturated fatty acids (PUFAs), which are integral to the structural integrity of the sperm plasma membrane (Sing et al., 1018).

Antifreeze proteins (AFPs), a class of ice-binding proteins found in extremophile organisms like fish and insects, have emerged as promising bio-cryoprotectants (Białkowska et al., 2020). By inhibiting ice recrystallization and lowering the freezing point of extracellular fluids through thermal hysteresis, AFPs prevent intracellular ice crystal formation, a major contributor to cryoinjury. Unlike conventional cryoprotective agents such as glycerol or dimethyl sulfoxide (DMSO), AFPs exert non-colligative effects, interacting directly with ice or cellular membranes, thereby offering more targeted and less cytotoxic protection (Duman, 2015).

Among the key components of the sperm membrane, lysophosphatidylcholine (LPC) stands out as a structurally significant phospholipid, contributing to membrane fluidity and acting as a substrate for lipid signaling pathways. During cryopreservation, LPC and other phospholipids undergo oxidative degradation, compromising the biophysical properties of the membrane. Therefore, preserving the functional integrity of LPC is critical to sustaining post-thaw sperm quality (Ghania et al., 2025).

Advancements in computational chemistry have made it feasible to simulate and predict protein-ligand interactions at the molecular level. Molecular docking offers insights into the spatial complementarity and binding affinities between biomolecules. In the context of cryopreservation, simulating the interaction between AFPs and LPC can provide mechanistic clues into how AFPs stabilize sperm membranes during freezing stress (Roche et al., 2015; Vajda et al., 2006).

In this study, we employed molecular docking approaches to evaluate the binding behavior of lysophosphatidylcholine with a series of AFPs derived from marine organisms. Using AutoDock 4.2, docking simulations were conducted, followed by structure visualization and interaction analysis using PyMOL. Energy refinement was carried out through semi-empirical calculations using the PM6 method in Gaussian 09. This integrative computational investigation aims to elucidate the structural compatibility and potential cryoprotective role of AFPs at the membrane interface, with implications for improving buffalo sperm cryopreservation protocols.

MATERIALS AND METHODS

PROTEIN AND LIGAND SELECTION AND PREPARATION

A diverse set of ten antifreeze proteins (AFPs), predominantly type II and III isoforms, were selected for this study based on their structural availability and relevance to thermal hysteresis activity. Protein structures were retrieved in .pdb format from the RCSB Protein Data Bank (PDB). The selected proteins included type III AFPs such as 1KDE, 1B7J, 1HG7, 1UCS, 2SPG, 4UR6, and 2PY2, and type II AFPs including 2AFP and 1MSI, as well as the winter flounder AFP (1WFA).

The phospholipid lysophosphatidylcholine (LPC)a critical membrane constituent of buffalo (*Bubalusbubalis*) sperm was chosen as the ligand. Its structure was obtained from the PubChem database in .sdf format (PubChem CID: 5311264), and converted into .pdb format using Open Babel for compatibility with docking software.

PROTEIN STRUCTURES PROCESS

Prior to docking, protein structures were processed using AutoDock Tools v1.5.6. Polar hydrogens were added, non-polar hydrogens were merged, Gasteiger charges were assigned to ligands, and Kollman charges were assigned to proteins. All bound water molecules and heteroatoms were removed to prevent interference with the docking calculations. Both protein and ligand files were finally saved in PDBQT format.

MOLECULAR DOCKING PROTOCOL

Molecular docking simulations were performed using AutoDock 4.2 employing the Lamarckian Genetic Algorithm (LGA) for conformational search and binding energy estimation. For each protein-ligand pair, the docking grid was centered over the entire protein to allow unbiased exploration of potential binding sites, with grid dimensions adjusted to encompass the full protein surface. The grid spacing was set at 0.375 Å, and each docking run was executed with 10 independent runs and a population size of 150, generating 2.5 million evaluations per run.

Each docking result produced a series of conformers ranked by binding energy. The lowest-energy conformation was selected for each protein-ligand complex and subjected to further analysis.

VISUALIZATION AND INTERACTION ANALYSIS

Post-docking visualization and analysis of protein-ligand interactions were conducted using PyMOL (v2.3). Hydrogen bonding, hydrophobic interactions, and positioning of the ligand within surface grooves or pockets of the AFPs were carefully examined. Structural alignments were performed where necessary to observe commonalities across AFP types.

It is installed from https://pymol.en.softonic.com/. It is used to visualize protein structure,ligandandalso complexwhichispreparedfromAutodock4. Inpymol,Clickon file menu, move on open bar from where a protein, our ligand or complex in pdb taken. Now our structure is visualized on pymol interface. On the right side of Pymol interface, therearefouroptionsseenonbar.ThesearеAction,Hide,Show,LabelandColour.After preparing the structure to use all these options, the resulting structure is saved by moving on file menu and clicking on export image as PNG.

ENERGY OPTIMIZATION AND BINDING AFFINITY CALCULATIONS

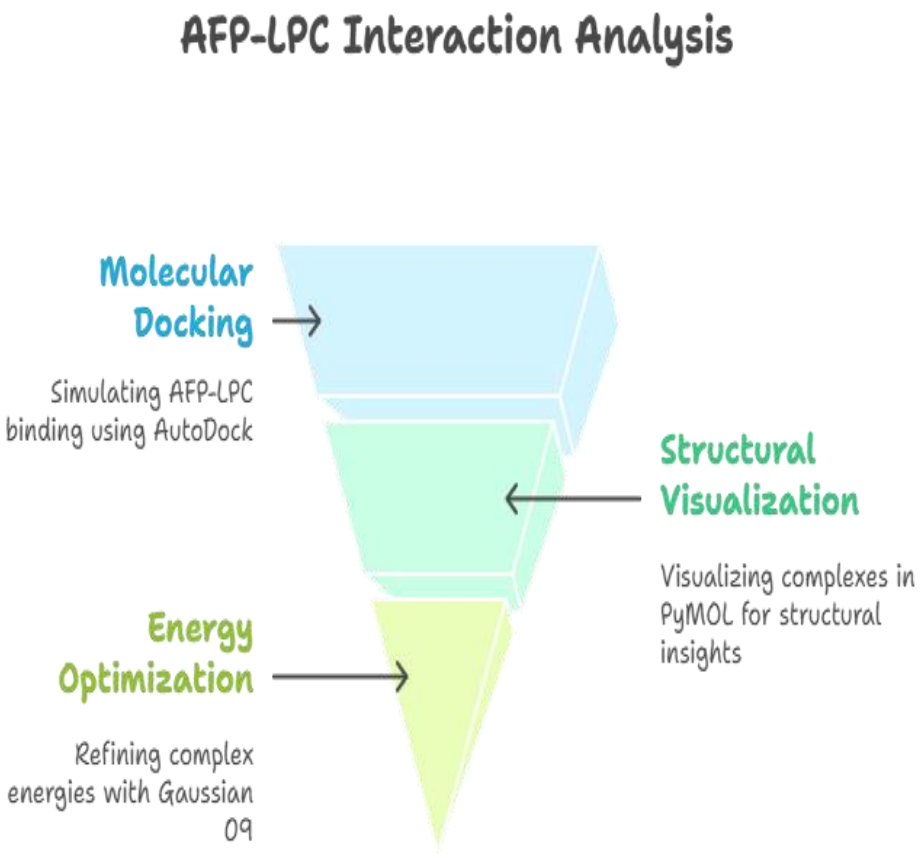
To refine and validate the docking results, energy minimizations were performed using Gaussian 09 with the PM6 semi-empirical method. The optimized geometries of both the docked complex and the individual components (protein and ligand) were calculated to derive more accurate interaction energies.Energies were first obtained in atomic units (a.u.) and subsequently converted to kcal/mol. All values were tabulated for comparative analysis, allowing assessment of the relative stability of each docked complex.

PROTEINS USED IN THE STUDY

IKDE is a type III antifreeze protein that is found in the North Atlantic Ocean. It is an isoform of the HPLC12 mutant. IMSIisan antifreezeglycoprotein. IUCS is a globular and type III antifreeze protein. 4UR6 is atype IIIantifreezeprotein found inZoarcesviviparous (ZvAFP6). IB7J isalsoatypeIIIantifreezeproteinwith aglobularstructure. 2SPG is a typeIIIantifreezeprotein thatbelongsto the isoformHPLC12T15S and is extracted from Zoarcesamericanus, which is a type of ocean pout fish. 2PY2 isatypeIIantifreezeproteinthat containssixchainsandhas136residues. IWFAs, referred to as winterflounderantifreezeproteins, live at 4°C. It has two chains with 38 residues. 2AFP is a typeIIantifreezeprotein that belongsto the lectinfamily.Becauseitsstructurein solution resembles that of that family. It has one chain with 129 residues. 1HG7 isatype IIIantifreezeproteinwith an HPLC12structureextractedfromoceanpout fishes. It has a chain with 66 residues.

LIGANDS USED IN THE STUDY

Ligands used in our study were obtained from PubChem. These ligands are essentially fatty acids and phospholipids present in buffalo spermatozoa. Ligands such as Docohexanoic Acid have a structure that is deduced from PubChem, *i.e.*,PubChem CID 445580, and this structure contains six double bonds, which are cis to each other. Its molecular formula is $C_{22}H_{32}O_2$. The ligand linoleic acid is a polyunsaturated fatty acid, PubChem CID 5280450, which has two double bonds. Its molecular formula is $C_{18}H_{32}O_2$, whereas lysophosphatidylcholine is a phospholipid with PubChem CID 5311264. Its molecular formula is $C_{10}H_{22}NO_7P$. Eicosapentanoic acid is a polyunsaturated fatty acid containing six double bonds. Its structure is obtained from PubChem CID 446284. Its molecular formula is $C_{20}H_{30}O_2$, whereas docosapentanoic acid contains five double bonds; thus, it is a polyunsaturated fatty acid with PubChem CID 5497182. Its molecular formula is $C_{22}H_{34}O_2$.



GRAPHICAL ABSTRACT,MOLECULAR DOCKING ANALYSIS OF ANTIFREEZE PROTEINS WITH LYSOPHOSPHATIDYLCHOLINE FROM BUFFALO SPERM

RESULTS

To study the interaction between proteins and ligands, a autodock4 software is used.Protein pdb files obtained from RCSB protein data bank. Ligands are obtainedfrom pubchem and save in sdf file. Since ligands are used in pdb format in docking so, it is compulsoryto convert sdf file into pdb files by using software open babble. So first of all I take the protein 1kde and it is docked against a ligand that is lysophosphatidylcholinepresentinbuffalosperm.

BINDING MODES OF LYSOPHOSPHATIDYLCHOLINE WITH ANTIFREEZE PROTEINS

Lysophosphatidylcholine is a phospholipid having PubChem CID 5311264. Its molecular formula is C10H22NO7P. Molecular docking simulations revealed diverse binding patterns between lysophosphatidylcholine (LPC) and the panel of ten antifreeze proteins (AFPs). Notably, LPC exhibited a preference for surface grooves, hydrophobic clefts, and charged patches across different AFP isoforms, suggesting broad structural compatibility. These interaction zones were primarily stabilized via hydrogen bonding, electrostatic interactions, and van der Waals forces, indicative of favorable ligand accommodation and binding plasticity.

For instance, in the docking complex of 1KDE (Fig. 1a), a type III AFP from North Atlantic ocean pout, LPC nestled into a shallow hydrophobic groove flanked by polar residues, establishing hydrogen bonds with backbone amide groups and side chains such as serine and glutamine. The interaction surface showed a complementary topology that allowed the ligand's phosphate and choline moieties to remain solvent-accessible while anchoring its lipid tail within the protein core. Similarly, the docking of LPC with 1WFA (Fig. 1b), a winter flounder-derived AFP, revealed binding within a concave β-sheet region stabilized by hydrogen bonding and electrostatic interactions. The ligand occupied a site overlapping with previously reported ice-binding motifs, raising the possibility of dual membrane- and ice-interacting functionality.

AFP structures such as 2AFP (Fig. 1c) and 2PY2 (Fig. 1d) both representing type II isoforms, also demonstrated high-affinity LPC binding. In these cases, the ligands aligned along longitudinal protein clefts, engaging with cysteine-rich regions known for structural rigidity and disulfide-mediated stability. Interestingly, the observed binding patterns suggest that both type II and III AFPs are structurally versatile in accommodating lipid-based ligands like LPC.

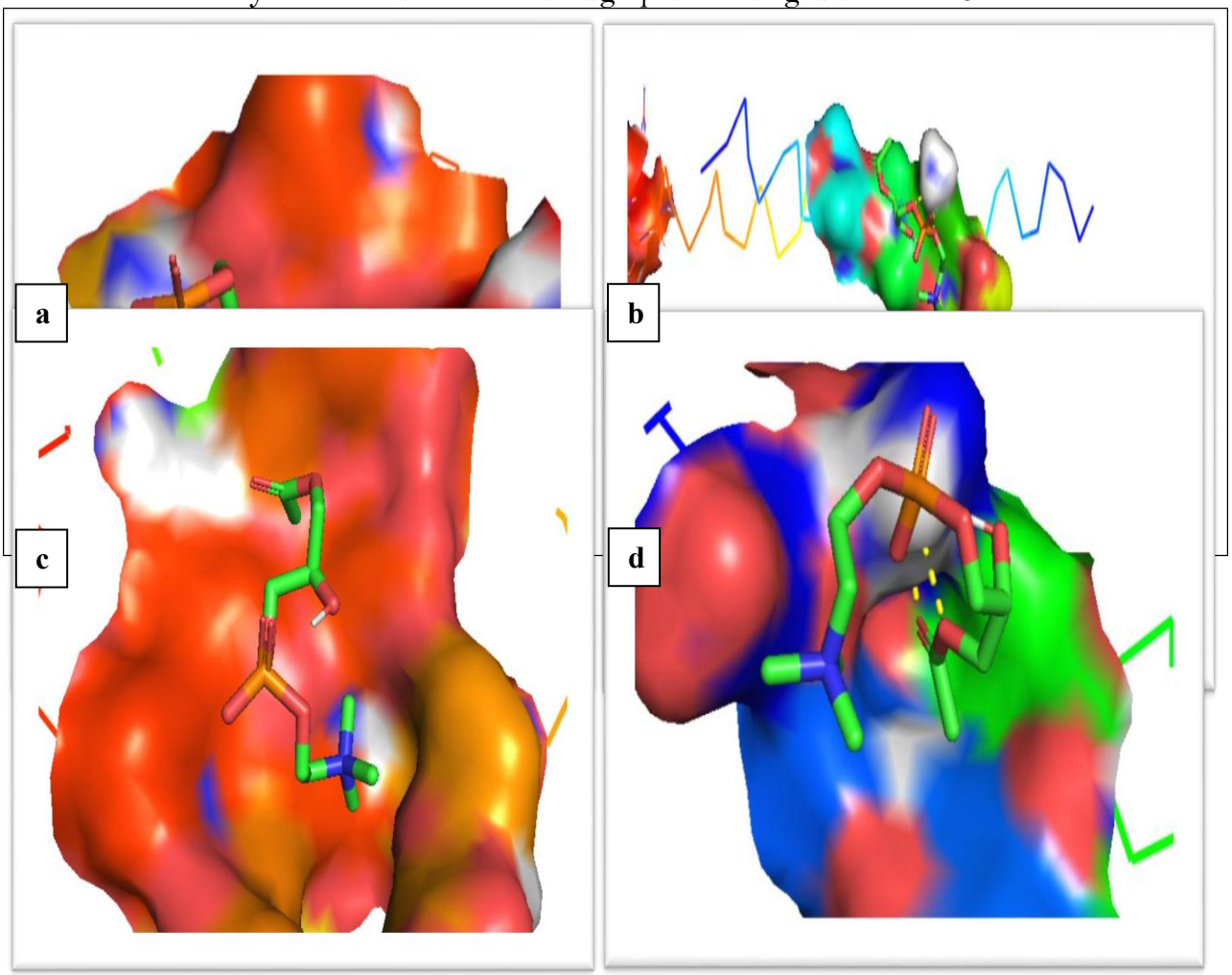


FIG. 1 (1a-1d): Docking of Type III Antifreeze Protein of North Atlantic Ocean Pout (1KDE) (a), Antifreeze protein (1WFA) (b), Type II Antifreeze Protein (2AFP) (c), Type II Antifreeze Protein(2PY2) (d) against Lysophosphatidylcholine

DOCKING ENERGIES AND INTERACTION PROFILES

Lysophosphatidylcholine is a phospholipid having PubChem CID 5311264. Its molecular formula is C10H22NO7P (Fig. 2a). Binding energies obtained from AutoDock and refined via PM6 semi-empirical calculations revealed a spectrum of affinities across the AFP panel (Table 1). The lowest binding energy was observed in the 2AFP-LPC complex, with a $\Delta E_binding$of -86.53 kcal/mol, indicating a highly stable interaction. Other notable interactions included 1WFA-LPC (-47.71 kcal/mol) and 2PY2-LPC (-17.80 kcal/mol), demonstrating strong thermodynamic favorability.

Conversely, certain AFPs such as 2SPG (Fig. 2b) and 1B7J (Fig. 2c) showed anomalously positive binding energy values (e.g., +289.68 kcal/mol and +169.20 kcal/mol, respectively), suggesting steric mismatch or unfavorable conformations during docking. These outliers likely reflect limitations in docking accuracy or conformational flexibility not fully accounted for in rigid-receptor docking protocols. These docking energy profiles align with visual inspections from PyMOL, where tighter ligand-protein interfaces and more extensive hydrogen bonding networks were consistently observed in low-energy complexes.

In all stable complexes, the glycerophospholipid head group of LPC remained outwardly oriented,

mimicking its natural membrane orientation, while the hydrophobic tail integrated within protein crevices, further supporting the hypothesis of membrane-like anchorage.

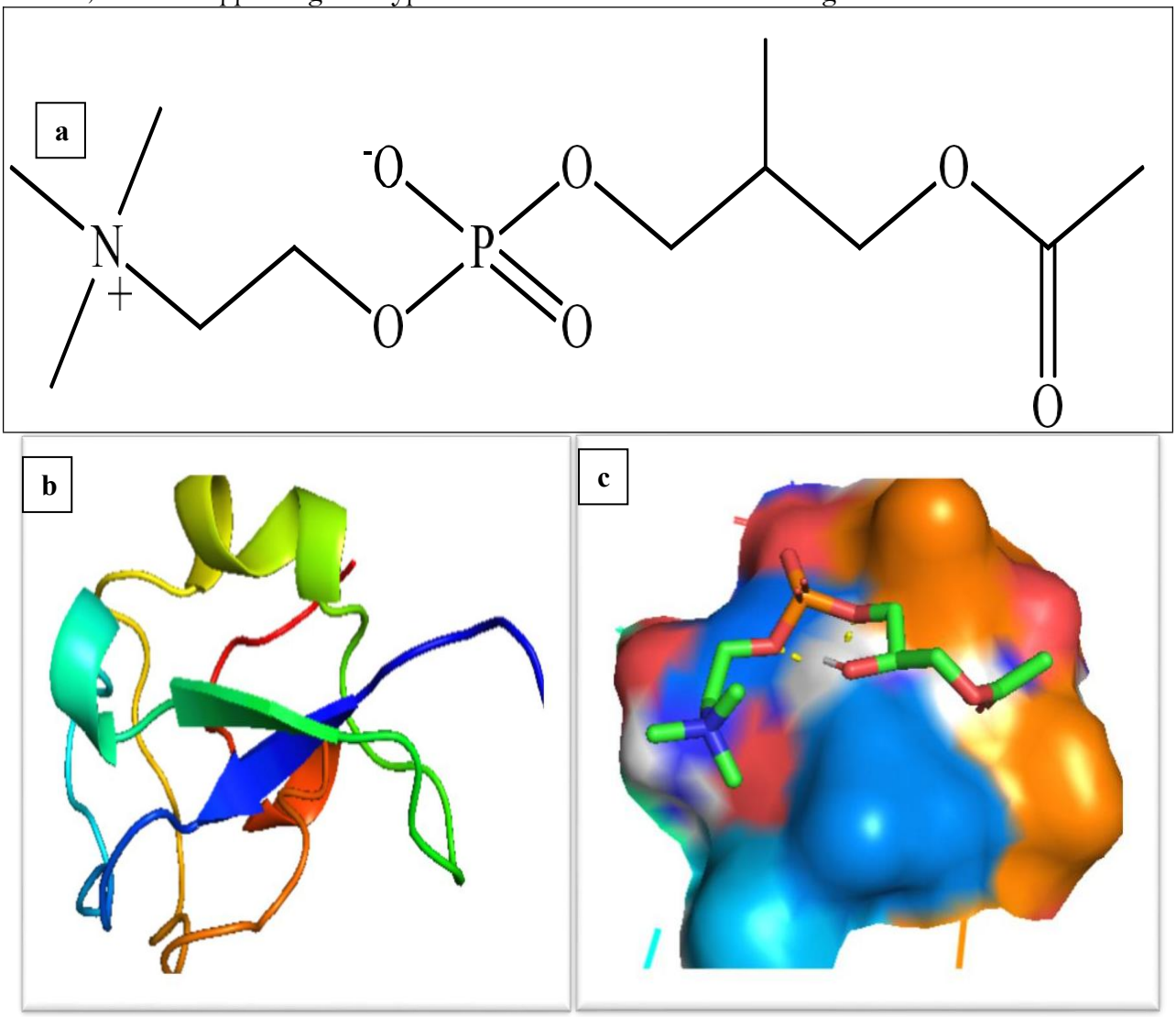


FIG. 2 (2a-2c): Structure of Lysophohatidylcholine by ChemDraw (**a**),Docking of Type III antifreeze protein (2SPG) (**b**), Type III antifreeze protein (1B7J) (**c**) against Lysophosphatidylcholine

OPTIMIZATION AND ENERGY CALCULATION

Open autodock 4, open the complex in pdbqt , go to option edit and add hydrogen. Then move on option Misc to repair the missing atom. The proteins in complexes also repair in such a way that the amino acids which interact with ligands, are retained in the complex and all other amino acids are deleted in autodock 4. At the end, the file is saved in pdb. In the next step, there is a optimization of these complexes. For optimization, a software Avogadro is used. For each complex, we have to optimize its ligand and its protein separately by using the following basis set:

opt pm6 at guassian 09. In this way, their energies are calculated for complex, its ligand and protein. It means, we have to calculate three energies one for complex, 2nd and 3rd for its ligand and protein respectively. These energies are noted in a.u. unit. We have to convert it into Kcal/mol. The energies of complexes are compared by the sum of energies of Proteins and ligands one by one (Table 1).

TABLE 1: THE DIFFERENCE BETWEEN SUM OF ENERGY OF LIGAND AND PROTEIN AND ENERGIES OF COMPLEX

Sr No	Protein ID	Protein Name	Ligand	BindingEnergyof Complex
1	1KDE	TypeIIIAntifreezeprotein	Lysoposhphatidylcholine	19.23kcal/mole
2	1UCS	TypeIIIAntifreezeprotein	Lysoposhphatidylcholine	-15.87kcal/mole
3	1B7J	TypeIIIAntifreezeprotein	Lysoposhphatidylcholine	169.2kcal/mole
4	1WFA	Winter FlounderAntifreezeprotein	Lysoposhphatidylcholine	-47.71kcal/mole
5	2AFP	Type IIAntifreezeprotein	Lysoposhphatidylcholine	-86.525kcal/mole
6	2PY2	Type IIAntifreezeprotein	Lysoposhphatidylcholine	-17.795kcal/mole
7	2SPG	TypeIIIAntifreezeprotein	Lysoposhphatidylcholine	289.68kcal/mole
8	2PY2	Type IIAntifreezeprotein	Docohexanoic acid	60.055kcal/mole
9	1WFA	Winter FlounderAntifreezeProtein	Docohexanoic acid	-38.085kcal/mole
10	1KDE	TypeIIIAntifreezeprotein	Docohexanoic acid	-0.379kcal/mole

DISCUSSION

This study provides computational evidence supporting the potential of antifreeze proteins (AFPs) to stabilize sperm membrane lipids during cryopreservation by directly interacting with lysophosphatidylcholine (LPC), a key component of the buffalo sperm plasma membrane.

BINDING ENERGY AS INDICATOR OF AFFINITY

The docking results demonstrated variable but often favorable binding energies between AFPs and LPC. Notably, type II AFPs such as 2AFP (−86.53 kcal/mol) and type III AFPs like 1WFA (-47.71 kcal/mol) formed the most stable complexes. These values indicate strong and spontaneous binding, supporting the hypothesis that AFPs may anchor or shield phospholipids

under cold stress.

Alpha-fetoprotein (AFP), a 69-kilodalton glycoprotein related to the albumin family, plays dual roles as a molecular transporter and a growth regulator (Nambier, 2021). Clinically, it is well-known as a biomarker for both fetal abnormalities and cancer. On the other hand, lysophospholipids (LPLs) are small, bioactive lipids originating from the cell membrane, made up of a single fatty acid chain (such as palmitic or oleic acid) and a polar headgroup (Meyerand Heringdorf, 2022; Omi et al., 2021). These lipids, found exclusively in vertebrates, include key molecules like sphingosine-1-phosphate and lysophosphatidic acid, along with their derivatives (choline, ethanolamine, serine, or inositol-based) (J Mizejewski, 2017).

FUNCTIONAL COMPLEMENTARITY EVIDENCED BY LPC-AFP INTERACTION

Molecular docking revealed that lysophosphatidylcholine (LPC) consistently nestled into surface grooves and hydrophobic clefts of antifreeze proteins (AFPs), forming a range of non-covalent interactions, most notably hydrogen bonds and van der Waals contacts. Notably, the polar headgroup of LPC remained solvent-exposed, while its hydrophobic acyl chain buried itself within the protein's core. This orientation echoes LPC's natural membrane conformation, suggesting a structural complementarity that allows AFPs to interface with membrane-like environments. Such lipid recognition patterns align with previous reports of AFP-lipid interactions, underscoring their membrane-affine nature during cryostress conditions (Kar et al., 2016; Smith and Haymet, 2023; Khan et al., 2022; Waqas, 2025).

MEMBRANE STABILIZATION: A CRYOPROTECTIVE SYNERGY

Beyond their canonical ice-binding properties, the observed interactions point toward a novel functional axis: membrane stabilization. During cryopreservation, LPCs are prone to phase transitions and lipid peroxidation, which compromise membrane fluidity and cellular viability (LaVelleet a., 2023; Aly et al., 2025). The binding of AFPs to LPC may act as a molecular shield, minimizing lipid disarray, buffering oxidative stress, and maintaining bilayer cohesion. By preserving LPC integrity, AFPs could mitigate reactive oxygen species (ROS)-induced lipid degradation, thereby enhancing post-thaw sperm viability and motility (Almeida, et al., 2021; Shah and Andrabi. 2021; Venkatesh et al., 2024).

REVISITING AFP FUNCTIONALITY: BEYOND ICE INHIBITION

Traditionally, AFPs are lauded for their ice recrystallization inhibition (IRI) and thermal hysteresis (TH) properties, critical for preventing ice damage during freezing (Gao et al., 2025; Ghalamara et al., 2022). However, our findings suggest that AFPs possess a functional duality: while externally modulating ice crystal dynamics, they may internally stabilize cellular membranes through lipid-binding. This dual mechanism could be particularly beneficial for buffalo spermatozoa, which possess high levels of unsaturated lipids, rendering them more susceptible to cryoinjury (Singh et al., 2022; Venkatesh et a;., 2024; Kabir et al., 2024). This synergistic role of AFPs not only enhances cryoprotection but also opens new avenues for bioengineering next-generation cryopreservation agents.

ANOMALIES AND STRUCTURAL FLEXIBILITY CONSIDERATIONS

Interestingly, some AFP variants (e.g., 2SPG and 1B7J) yielded unfavorable binding energies, indicating potential incompatibility with LPC. These deviations may stem from the limitations of rigid docking algorithms, which often overlook protein-ligand dynamics and conformational plasticity (Karimi et al., 2025; Khalil et al., 2025; Hassan et al., 2024). Alternatively, these proteins may naturally exhibit low affinity for LPC due to sequence or structural constraints. To resolve this, advanced molecular dynamics simulations are warranted, which can better

capture the thermodynamically relevant binding modes and lipid-anchoring behavior under physiologically relevant conditions.

In summary, our results support the rational inclusion of AFPs in cryopreservation media to enhance lipid membrane resilience in sperm cells, particularly in species with high PUFA content such as buffalo.

CONCLUSION

The molecular docking analysis conducted in this study reveals that antifreeze proteins (AFPs) possess an intrinsic ability to interact with lysophosphatidylcholine (LPC), a phospholipid critical for maintaining the integrity of buffalo sperm membranes during cryopreservation. Several AFP isoforms, particularly type II (2AFP) and type III (1WFA), exhibited stable and energetically favorable interactions with LPC, engaging through hydrophobic and electrostatic contacts within structured surface pockets. These interactions likely contribute to the preservation of lipid bilayer architecture, reduce phase transitions, and protect against oxidative membrane damage during freezing and thawing. Importantly, this suggests that AFPs function not only as extracellular ice-binding agents but also as intracellular lipid stabilizers, thereby enhancing their efficacy as dual-function cryoprotectants. The application of such proteins in cryopreservation media could significantly improve post-thaw sperm quality, especially in species like buffalo that are prone to cryogenic damage due to their lipid-rich spermatozoa. These findings provide a computational foundation for future experimental validation and pave the way for bioengineered, AFP-enriched freezing extenders in livestock biotechnology.

DECLARATIONS

ETHICS APPROVAL

The Institutional Ethics and Guideline Committee of the University of Lahore, Lahore Pakistan has allowed all the protocols used in this experiment. All the experimental methods of this study followed all the appropriate guidance and regulations.

CONSENT FOR PUBLICATION

All subjects gave their "informed consent" for the publication of details within the text ("informed consent") to be published in the above Journal and Article. Written "informed consent" was obtained from all authors for the publication of this manuscript.

AVAILABILITY OF DATA AND MATERIALS

The data generated are provided within the manuscript and will be available from author at reasonable request

COMPETING INTEREST

All authors declare that there are no competing interests.

FUNDING

Not applicable

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